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Inhibition of NADPH-Cytochrome P450 Reductase and Glyceryl Trinitrate Biotransformation by Diphenyleneiodonium Sulfate

John J. McGuire, Diane J. Anderson,* Bernard J. McDonald,* Ramani Narayanasami† and Brian M. Bennett*‡*

*Department of Pharmacology and Toxicology, Queen's University, Kingston, Ontario, Canada K7L 3N6; and †The University of Texas Health Science Center at San Antonio, San Antonio, TX 78284-7760, U.S.A.

ABSTRACT. We reported previously that the flavoprotein inhibitor diphenyleneiodonium sulfate (DPI) irreversibly inhibited the metabolic activation of glyceryl trinitrate (GTN) in isolated aorta, possibly through inhibition of vascular NADPH-cytochrome P450 reductase (CPR). We report that the content of CPR represents 0.03 to 0.1% of aortic microsomal protein and that DPI caused a concentration- and time-dependent inhibition of purified cDNA-expressed rat liver CPR and of aortic and hepatic microsomal NADPH-cytochrome *c* reductase activity. Purified CPR incubated with NADPH and GTN under anaerobic, but not aerobic conditions formed the GTN metabolites glyceryl-1,3-dinitrate (1,3-GDN) and glyceryl-1,2-dinitrate (1,2-GDN). GTN biotransformation by purified CPR and by aortic and hepatic microsomes was inhibited > 90% after treatment with DPI and NADPH. DPI treatment also inhibited the production of activators of guanylyl cyclase formed by hepatic microsomes. We also tested the effect of DPI on the hemodynamic-pharmacokinetic properties of GTN in conscious rats. Pretreatment with DPI (2 mg/kg) significantly inhibited the blood pressure lowering effect of GTN and inhibited the initial appearance of 1,2-GDN (1–5 min) and the clearance of 1,3-GDN. These data suggest that the rapid initial formation of 1,2-GDN is related to mechanism-based GTN biotransformation and to enzyme systems sensitive to DPI inhibition. We conclude that vascular CPR is a site of action for the inhibition by DPI of the metabolic activation of GTN, and that vascular CPR is a novel site of GTN biotransformation that should be considered when investigating the mechanism of GTN action in vascular tissue. BIOCHEM PHARMACOL **56**;7:881–893, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. biotransformation; diphenyleneiodonium; flavoproteins; glyceryl trinitrate; NADPH-cytochrome P450 reductase; vascular smooth muscle

Biotransformation of GTN§ has been shown to be mediated by several proteins [1], including reduced unliganded hemoproteins [2], vascular glutathione *S*-transferases [3, 4], and the cytochromes P450-NADPH-cytochrome P450 reductase system [5–7]. There is also an unidentified microsomal protein isolated from bovine coronary artery that mediates NO formation from GTN [8]. In addition to the formation of the denitrated metabolites 1,2-GDN and 1,3-GDN, both NO_2^- and NO may be formed during the biotransformation reaction. However, the low vasodilator potency of NO_2^- indicates that it is of little importance in mediating the pharmacological actions of organic nitrates.

Hepatic and aortic microsomal biotransformation of GTN to 1,3-GDN and 1,2-GDN has been shown to be NADPH dependent, inhibited by cytochrome P450 inhibitors, such as carbon monoxide and SKF 525A, and increased in microsomes from phenobarbital-treated rats [6, 7]. Furthermore, rat hepatic microsomal biotransformation of GTN results in the formation of an activator of guanylyl cyclase (presumably NO) [9]. Whether the vascular cytochromes P450-NADPH-cytochrome P450 reductase system mediates bioactivation of GTN remains controversial, since classical inhibitors of cytochrome P450 such as SKF 525A [9–11], cimetidine [9], and metyrapone [11] have no effect on GTN-induced vasodilation. In contrast, the cytochrome P450 substrate 7-ethoxyresorufin inhibits GTN-induced relaxation, cyclic GMP accumulation and aortic biotransformation of GTN [12].

Because NADPH-cytochrome P450 reductase is essential for cytochrome P450 activity, inhibition of this flavoprotein might be expected to inhibit the pharmacological actions of GTN. DPI is known to inhibit a number of flavoproteins including the NADPH oxidase system of neutrophils and macrophages [13, 14], xanthine oxidase

‡ Corresponding author. Tel. (613) 533-6473; FAX (613) 533-6412; E-mail: bennett@post.queensu.ca

§ *Abbreviations:* DPI, diphenyleneiodonium sulfate; FMN, flavin mononucleotide; FMO, flavin-containing monooxygenase; 1,3-GDN, glyceryl-1,3-dinitrate; 1,2-GDN, glyceryl-1,2-dinitrate; GTN, glyceryl trinitrate; IDP, iodonium diphenyl; MAP, mean arterial pressure; and NO, nitric oxide.

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[15], and macrophage NO synthase [16]. IDP, an analog of DPI, is an irreversible inhibitor of bovine liver NADPH-cytochrome P450 reductase [17]. In a previous study, we found that DPI irreversibly inhibits GTN-induced relaxation and cyclic GMP accumulation in isolated rat aorta and the selective biotransformation of GTN to 1,2-GDN [18]. DPI also inhibits the enantioselectivity of isoidide dinitrate action by selectively inhibiting biotransformation of, and cyclic GMP accumulation and relaxation induced by, the more potent D-enantiomer [19]. In addition, DPI has no effect on GTN or isoidide dinitrate biotransformation in aortic cytosol, suggesting that the site of action of DPI for the inhibition of organic nitrate biotransformation is in the microsomal fraction of vascular tissue [19]. We proposed that DPI may be reduced by NADPH-cytochrome P450 reductase to form a radical that then forms an adduct with either the heme (cytochrome P450), the flavin cofactors of NADPH-cytochrome P450 reductase, or amino acids of either cytochrome P450 or NADPH-cytochrome P450 reductase [18].

In the current study, we used kinetic analysis to assess whether NADPH-cytochrome P450 reductase activity was inhibited by DPI through an irreversible mechanism, and tested the effect of DPI on mitochondrial/microsomal NADH-cytochrome b_5 reductase and the flavin-containing NADH-ubiquinone oxidoreductase (Complex 1), a component of the electron transport chain in mitochondria. We also examined the effect of DPI on GTN biotransformation by aortic and hepatic microsomes, and by purified NADPH-cytochrome P450 reductase. To assess the *in vivo* significance of DPI inhibition of GTN action, we tested the effect of DPI on hemodynamic and pharmacokinetic properties of GTN in conscious rats.

MATERIALS AND METHODS

Materials

DPI was purchased from Colour Your Enzyme. Stock solutions of DPI (1 mM) were prepared in distilled water for *in vitro* studies and in 5% dextrose solution for *in vivo* studies. GTN was obtained as a solution (TRIDIL®, 5 mg/mL) in ethanol, propylene glycol, and water (1:1:1.33) from DuPont Pharmaceuticals. 1,2-GDN and 1,3-GDN were prepared by acid hydrolysis and purified by thin-layer chromatography [20]. The purified 1,2-GDN and 1,3-GDN were quantified by the method of Dean and Baun [21] as modified by Bennett *et al.* [22]. Isosorbide-2-mononitrate was a gift from Wyeth Ltd. β-NADPH, β-NADH, rotenone, and bovine heart cytochrome *c* were purchased from the Sigma Chemical Co. All other chemicals were of at least reagent grade and were obtained from a variety of sources.

Microsomal Protein Preparation

The microsomal fractions from rat aorta and liver were prepared by standard differential centrifugation techniques. Liver microsomal protein was prepared from individual male Sprague–Dawley rats (275–325 g) [6]. Aortic microsomes were prepared from the pooled aortae of 20–40 rats [7]. Aortic microsomes (105,000 *g* pellet) were resuspended in 50 mM potassium phosphate buffer (pH 7.7) containing 0.1 mM EDTA. Protein concentrations were determined by the method of Lowry *et al.* [23].

Mitochondria Isolation

The mitochondrial fraction from rat aorta and liver was prepared using the isolation method of Kukielka *et al.* [24] with the modification that livers from individual rats were perfused with ice-cold 10 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA prior to homogenization (10%, w/v) in the same buffer. Mitochondria from rat aorta were obtained from a modified microsomal preparation using 30 rats (see above). The modifications were the use of the microsomal homogenization buffer [7] and the inclusion of the 800 *g* centrifugation step.

Identification of NADPH-Cytochrome P450 *Reductase*

Purified cDNA-expressed rat liver NADPH-cytochrome P450 reductase (a gift from Dr. B. S. S. Masters, The University of Texas Health Science Center at San Antonio) [25, 26] and aortic microsomal and/or hepatic microsomal proteins were separated by electrophoresis on 10% SDS polyacrylamide gels. Proteins were transferred electrophoretically to PVDF membranes, and incubated overnight with an anti-rat liver NADPH-cytochrome P450 reductase antibody (also a gift from Dr. B. S. S. Masters); immunoreactive bands were visualized using enhanced chemiluminescence.

NADPH-Cytochrome P450 *Reductase Activity*

NADPH-cytochrome P450 reductase activity of purified enzyme and microsomal fractions was measured by following the NADPH-dependent reduction of bovine heart cytochrome *c* [27]. Samples (1 mL) contained 50 mM potassium phosphate buffer (pH 7.7), 0.1 mM EDTA, 100 μM NADPH, 36 μM cytochrome *c* and either 2 nM purified NADPH-cytochrome P450 reductase, 110 μg/mL of hepatic microsomal protein, or 50 μg/mL of aortic microsomal protein. KCN (1 mM) was added to samples containing microsomal protein to inhibit possible contamination by mitochondrial cytochrome *c* oxidase. Reactions were initiated by the addition of protein, and A_{550} was measured using an HP 8451A diode array spectrophotometer. Reaction rates were calculated using the extinction coefficient for cytochrome *c* of 0.021 μM^{-1} cm^{-1}. In experiments examining the effect of DPI on NADPH-cytochrome P450 reductase activity, protein was preincubated at 25° with 100 μM NADPH and DPI (0.03 to 10 μM) for various times (15 sec to 5 min), prior to determining cytochrome *c* reductase activity.

NADH-Cytochrome c Reductase Activity

The activity of NADH-cytochrome b_5 reductase in intact mitochondria or microsomes was determined by measuring rotenone-insensitive NADH-cytochrome *c* reduction [24]. The activity of NADH-ubiquinone oxidoreductase was determined by measuring rotenone-sensitive NADH-cytochrome *c* reduction in mitochondria. Samples (1 mL) contained 0.2 M Tris–HCl buffer (pH 7.4), 50 μM cytochrome *c*, 1 mM KCN, 100 μM NADH, 10–30 μg of mitochondrial protein (or 0.1 mg of microsomal protein) and either rotenone (5 μM) or DPI (10 μM, 1/10 dilution of the preincubation sample concentration). Control samples (minus rotenone) contained 2.5% (v/v) ethanol (rotenone vehicle). In experiments examining the effect of DPI, protein was preincubated with 100 μM NADH and 100 μM DPI (100 μL vol.) for 15 min prior to determining cytochrome *c* reductase activity. Since NADH-ubiquinone oxidoreductase activity is associated with the inner membrane of mitochondria, to measure exogenous cytochrome *c* reduction by this enzyme component first required permeabilizing the mitochondria to NADH and cytochrome *c*. Intact mitochondria were treated with 0.033% Triton X-100/mg of mitochondrial protein, which was found to increase rotenone-sensitive activity without a loss of total NADH-cytochrome *c* reductase activity.

Kinetic Analysis of Enzyme Inactivation

We have based the kinetic analysis of enzyme inactivation on the following enzyme reaction equation [28]:

$$E + I \underset{}{\overset{K_i}{\rightleftarrows}} E{\cdot}I \overset{k_3}{\rightarrow} E \cdot I^* \quad (1)$$

where E represents active enzyme, I represents inhibitor, E · I represents the reversible enzyme–inhibitor complex, E · I* represents the irreversible enzyme–inhibitor complex, K_i is the apparent equilibrium constant for formation of E · I, and k_3 is the rate of conversion of E · I to E · I*. To determine the values for apparent K_i and k_3, we used the method of Kitz and Wilson [28] to solve the equation:

$$1/k_{\text{inactivation}} = 1/k_3 + (K_i/k_3) \cdot (1/[\text{I}]). \quad (2)$$

GTN Biotransformation Studies

Purified expressed rat liver NADPH-cytochrome P450 reductase (5 μg/mL of protein; 60 nM NADPH-cytochrome P450 reductase) was incubated with 0.2 μM GTN and 1 mM NADPH in 50 mM potassium phosphate buffer (pH 7.7) containing 0.1 mM EDTA. Incubations were performed in an Instrumentation Laboratory 237 tonometer at 37° under either aerobic (normal atmospheric oxygen) or anaerobic (humidified N_2 at a flow rate of 250 mL/min) conditions. An aliquot (1 mL) was taken immediately after the addition of GTN and then after 1 hr of incubation. Extraction of GTN and the GTN metabolites 1,2-GDN and 1,3-GDN and quantitation by megabore capillary column gas liquid chromatography were performed as described [6]. In experiments examining inhibition of GTN biotransformation by DPI, aortic microsomes (0.5 mg/mL of protein), hepatic microsomes (0.9 mg/mL of protein), or purified rat liver NADPH-cytochrome P450 reductase (60 nM) were treated with 100 μM NADPH and either 10 μM DPI or diluent for 10 min (hepatic microsomes and purified reductase) or 30 min (aortic microsomes) prior to the addition of 0.2 μM GTN and 1 mM NADPH. Hepatic microsomal biotransformation of GTN was determined over a 10-min time course. GTN biotransformation by aortic microsomes and by purified rat liver NADPH-cytochrome P450 reductase was assessed after a 1-hr incubation with GTN. Nonenzymatic denitration of GTN was determined by incubating samples in the absence of protein. NADH-dependent GTN biotransformation was assessed using the same procedures as NADPH-dependent GTN biotransformation under both aerobic and anaerobic conditions. Samples (1 mL) containing liver mitochondria (0.6 mg/mL of protein), aortic mitochondria (0.1 mg/mL of protein), or liver microsomes (0.6 mg/mL of protein) were treated with NADH (1 mM) and GTN (0.2 μM) for 10 min (liver proteins) or 60 min (aortic protein). In experiments to determine the effect of DPI on GTN metabolism, samples were preincubated with NADH (100 μM) and either DPI (10 μM) or diluent prior to initiating the reactions with GTN and NADH.

Guanylyl Cyclase Activity

The preparations of crude aortic guanylyl cyclase and enzyme assay conditions have been described [9]. In addition to the usual assay components, samples contained GTN (1 μM to 0.3 mM), DPI (10 μM), and either 1 mM NADPH or 1 mM NADH. Reactions were initiated by the addition of 18–23 μg of aortic supernatant protein and 21–30 μg of hepatic microsomal protein (20 pmol cytochrome P450) or 20–30 μg of mitochondrial protein. Reactions were terminated by the addition of 0.8 mL of 50 mM sodium acetate (pH 4.0) followed by heating at 90° for 3 min. Aliquots were assayed for cyclic GMP content by radioimmunoassay [29]. Aortic supernatant protein was determined by the method of Bradford [30]. Cytochrome P450 content of hepatic microsomes was measured by the method of Omura and Sato [31].

Surgical Preparation and Instrumentation of Rats

Male Sprague–Dawley rats (275–325 g) were anaesthetized with ketamine (Rogarsetic®, 70 mg/kg, i.p.) and xylazine (Rompun®, 10 mg/kg, i.p.). Polyethylene cannulas (PE 50) were inserted into the right jugular vein and left carotid artery and then exteriorized for delivery of intravenous injections and obtaining blood samples, respectively. An additional cannula was inserted into the abdominal aorta and exteriorized for measurement of mean arterial pressure

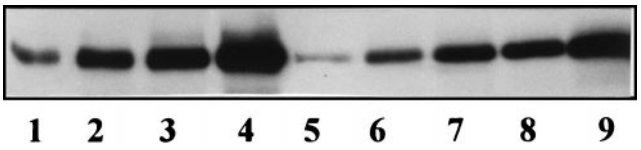

FIG. 1. Comparative immunoblot analysis of NADPH-cytochrome P450 reductases. Proteins were separated on 10% SDS polyacrylamide gels and transferred to PVDF membranes. NADPH-cytochrome P450 reductase was immunodetected using an antibody to rat liver NADPH-cytochrome P450 reductase and visualized by enhanced chemiluminescence. Lanes 1–4 contained 2, 4, 8, and 16 ng of purified rat liver NADPH-cytochrome P450 reductase, respectively. Lanes 5–7 contained 2, 4, and 8 μg of aortic microsomal protein, respectively. Lanes 8 and 9 contained 1 and 2 μg of hepatic microsomal protein, respectively.

and heart rate. To reduce infection, rats were given i.m. injections of streptomycin (40 mg/kg)/penicillin G (40,000 U/kg). Rats were allowed to recover for 48 hr before being used. Hematocrit was measured daily (no changes between days or treatments were found), and the dead volumes of cannulas were filled daily with heparinized saline (200 U/mL) and tested for patency. Heart rate and blood pressure were monitored through a Cobe CDX3 pressure transducer connected to a Micro-Med BP-100 Blood Pressure Analyser.

Experimental Protocols for In Vivo *Studies*

Forty-eight hours after surgery, rats were pretreated with diluent (5% dextrose in water, 1 mL/kg) for 15 min prior to the administration of GTN. After an additional 24 hr, the same rats were pretreated with DPI (2 mg/kg i.v. bolus) for 15 min prior to the administration of GTN. Blood pressure was monitored continuously, and the maximal decrease in MAP induced by each dose of GTN was used to calculate the percent change from baseline MAP. In the pharmacokinetic studies, blood samples (250 μL) were drawn from the carotid arterial cannula at set time points after a single i.v. bolus dose of GTN (1 mg/kg), added to ice-chilled microcentrifuge tubes, and centrifuged at 12,000 *g* for 30 sec; then the plasma was removed and frozen at −20° until analysis. Red blood cell fractions were kept on ice and infused back into the rats at the end of each experiment. Standard curves for GTN and its metabolites were prepared by spiking aliquots of rat plasma (100 μL) with known amounts of organic nitrates. Organic nitrates were quantitated by megabore capillary column gas–liquid chromatography [6]. In control experiments, consecutive treatment of rats as controls showed no significant differences in the levels of GTN, 1,2-GDN, or 1,3-GDN between days (data not shown).

RESULTS

NADPH-Cytochrome P450 *Reductase in Aortic Microsomes*

Based on immunoblot analysis (Fig. 1) and NADPH-cytochrome *c* reductase activity experiments, we have estimated the content of NADPH-cytochrome P450 reductase to represent between 0.03 and 0.1% of aortic microsomal protein. The content of NADPH-cytochrome P450 reductase in aortic microsomes appears to be 5- to 10-fold less than that found in hepatic microsomes, as determined by immunoblot comparisons (Fig. 1). We found that the activity of aortic microsomal preparations was 10.4 ± 3.1 nmol cytochrome *c* reduced/min/mg of protein (N = 8) and that the activity of purified cDNA-expressed rat liver NADPH-cytochrome P450 reductase was 16.5 ± 1.6 μmol cytochrome *c* reduced/min/mg of protein. The catalytic-center activity for the purified enzyme was 1316 ± 129 min^{-1}. Assuming an abundance of 0.065% of microsomal protein, the catalytic-center activity for the aortic microsomal enzyme was 1280 ± 382 min^{-1}.

Effect of DPI on NADPH-Cytochrome P450 *Reductase Activity*

Using purified NADPH-cytochrome P450 reductase, hepatic microsomes, or aortic microsomes, DPI caused a time-dependent and concentration-dependent inhibition of NADPH-cytochrome *c* reductase activity that appeared to follow first-order inactivation kinetics. Based on the data from the time-course inhibition experiments, the relationship between the rate of inactivation of enzyme activity and the concentration of DPI was plotted in accordance with equation (2) (Fig. 2). K_i/k_3 and $1/k_3$ values were determined from the parameters (slopes and y intercept) calculated by linear regression analysis for each preparation. Apparent K_i values of 0.20, 6.8, and 0.33 μM were determined for purified NADPH-cytochrome P450 reductase, hepatic microsomes, and aortic microsomes, respectively. Apparent k_3 values of 0.71 min^{-1}, 1.7 min^{-1}, and 1.3 min^{-1} were determined for purified NADPH-cytochrome P450 reductase, hepatic microsomes, and aortic microsomes, respectively. The lines describing data for the purified enzyme and aortic microsomes were not statistically different from each other, but both lines were different from that obtained for hepatic microsomes. There was no difference between $1/k_3$ values ($P > 0.05$, one-way analysis of variance), but statistical differences were found for comparisons of the K_i/k_3 values for purified enzyme and hepatic microsomes and for aortic microsomes and hepatic microsomes ($P < 0.001$, one-way ANOVA followed by Newman–Keuls post hoc test).

We also tested the effect of initial cytochrome *c* concentration (18–72 μM) on the inhibition of purified NADPH-cytochrome P450 reductase activity by DPI, and found that it had no significant effect on inhibition (data not shown). In addition, we tested the effect of initial NADPH concentration (1 μM–1 mM) on the inhibition of purified P450 reductase using 10 μM DPI and 36 μM cytochrome *c*. There was no apparent effect of DPI on the K_m value for NADPH (1–3 μM) but there was a decrease in V_{max}. In addition to using cytochrome *c* as a substrate, in which case electrons first pass from FAD and then through FMN, we

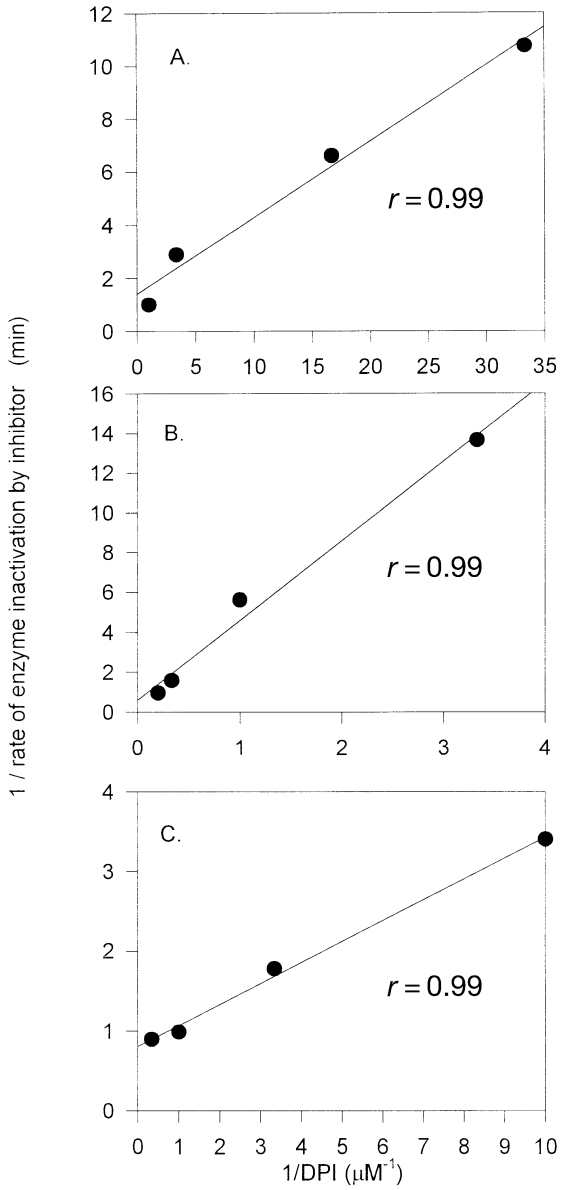

FIG. 2. Double-reciprocal plot of rate of inactivation ($k_{inactivation}$) of NADPH-cytochrome *c* reductase activity versus DPI concentration. Points on each graph represent the rates of inactivation determined from time–course inhibition data, with a solid line showing the results of linear regression analysis. The correlation coefficients (*r*) are shown for each line. The apparent K_i values for inhibition of enzyme activity by DPI were: (A) purified enzyme, 0.2 μM; (B) hepatic microsomes, 6.8 μM; and (C) aortic microsomes, 0.33 μM. The apparent k_3 values were: (A) purified enzyme, 0.71 min^{-1}; (B) hepatic microsomes, 1.7 min^{-1}; and (C) aortic microsomes, 1.3 min^{-1}. The measured rates of inactivation for each concentration of DPI (points on the graphs) represent the mean values determined from repeated experiments: (A) purified enzyme, N = 5; (B) hepatic microsomes, N = 4; and (C) aortic microsomes obtained from the pooled aortae of 40 rats, N = 1. For each experiment, the inhibition of NADPH-dependent cytochrome *c* reduction was determined for 4–6 time points at each concentration of DPI.

tested the effect of DPI on the reduction of potassium ferricyanide, in which electrons transfer directly from FAD. We found that DPI caused the same concentration-dependent and time-dependent inhibition of reductase activity in both cases (data not shown).

Effect of DPI on NADH-Cytochrome b_5 Reductase and Mitochondrial NADH-Ubiquinone Oxidoreductase (Complex 1)

The enzyme activities of NADH-cytochrome b_5 reductase and NADH-ubiquinone oxidoreductase were measured as rotenone-insensitive and rotenone-sensitive NADH-cytochrome *c* reductase activities, respectively (Table 1). The rotenone-insensitive NADH-cytochrome *c* reductase activities of intact liver and aortic mitochondria were not inhibited by DPI (Table 1) nor were those of liver and aortic microsomes (data not shown). DPI was found to inhibit the rotenone-sensitive NADH-cytochrome *c* reductase activity of liver mitochondria permeabilized to NADH and cytochrome *c* (Table 1).

Effect of DPI on the Biotransformation of GTN by Microsomes and by NADPH-Cytochrome P450 Reductase

The biotransformation of GTN by aortic microsomes and by purified expressed rat liver NADPH-cytochrome P450 reductase was assessed under both aerobic and anaerobic conditions. GTN biotransformation by purified expressed NADPH-cytochrome P450 reductase did not occur under aerobic conditions (data not shown). Under anaerobic conditions, incubation of the reductase with 1 mM NADPH and 0.2 μM GTN resulted in 1,2-GDN and 1,3-GDN formation (Table 2). Pretreatment of the reductase with 10 μM DPI for 10 min resulted in almost complete inhibition of GTN biotransformation activity (Table 2). Similarly, GTN biotransformation by aortic microsomes occurred only under anaerobic conditions, and pretreatment of aortic microsomal protein with 10 μM DPI for 30 min resulted in almost complete inhibition of GTN biotransformation (Table 2). In contrast to the relatively slow rate of denitration of GTN by NADPH-cytochrome P450 reductase and by aortic microsomes, biotransformation by hepatic microsomes was rapid (Fig. 3) with the disappearance of dinitrates starting at 2.5 min, reflecting the further metabolism to glyceryl mononitrates [6]. Pretreatment of hepatic microsomes with 10 μM DPI for 10 min abolished 1,2-GDN and 1,3-GDN formation. Treatment of microsomes or purified expressed NADPH-cytochrome P450 reductase using the same DPI treatment protocol abolished cytochrome *c* reductase activity in these preparations.

Effect of DPI on Hepatic Microsome-mediated Metabolic Activation of GTN

We found that treatment of hepatic microsomes with 10 μM DPI resulted in complete inhibition of hepatic microsome-mediated activation of aortic guanylyl cyclase by

TABLE 1. Effect of DPI on intact and permeabilized mitochondria NADH-cytochrome *c* reductase activity

	Cytochrome *c* reduced† (nmol/min/mg protein)		
	Liver		Aorta
Inhibitor treatment*	Intact	Permeabilized	Intact
Control	402 ± 63	489 ± 14	161
5 μM Rotenone	427 ± 120	405 ± 65‡	228
100 μM DPI + 100 μM NADH	460 ± 109	433 ± 30§	219

*Mitochondria from either liver (0.3 mg of protein/mL) or aorta (0.075 mg of protein/mL) were used either intact or permeabilized to NADH by Triton X-100 (0.033%/mg of protein) and were treated with either diluent [2.5% ethanol (v/v)], rotenone (5 μM), or pretreated with a combination of DPI (100 μM) plus NADH (100 μM) for 10 min prior to measuring NADH-dependent cytochrome *c* reduction (see Materials and Methods for details).

†Each value represents the mean ± SD [N = 5 (intact liver mitochondria); N = 4 (permeabilized liver mitochondria); N = 1 (pooled aortic mitochondria prepared from 28 rats)].

‡$P < 0.01$, significantly different from the control (repeated measures ANOVA; Newman–Keuls post hoc test).

§$P < 0.05$, significantly different from the control (repeated measures ANOVA; Newman–Keuls post hoc test).

GTN (Fig. 4). Based on the time course of inhibition (data not shown), the 10-min treatment of hepatic microsomes with 10 μM DPI would have been sufficient to cause greater than 95% inhibition of microsomal reductase activity. We also tested whether NADH could support GTN biotransformation by microsomes and mitochondria under aerobic and anaerobic conditions, and whether this biotransformation resulted in the formation of activators of guanylyl cyclase. NADH-dependent GTN biotransformation did not occur in hepatic mitochondria under anaerobic conditions or in either hepatic microsomes or aortic mitochondria under aerobic conditions. In contrast, incubation of hepatic mitochondria (aerobic) and hepatic microsomes (anaerobic) with NADH and GTN resulted in production of dinitrate metabolites, and this biotransformation was inhibited completely by DPI. However, this biotransformation did not result in the formation of activators of guanylyl cyclase.

In Vivo *Effects of DPI on GTN Hemodynamics and Pharmacokinetics*

We tested the effect of DPI on GTN-induced vasodilation and GTN pharmacokinetics in conscious rats (Fig. 5). After treatment with DPI, there was a significant reduction in the blood pressure lowering effect of GTN. Using the dose of GTN (1 mg/kg) that resulted in vasodilation that was inhibited significantly by DPI, we examined the time course of arterial plasma concentrations of GTN and its dinitrate metabolites for 1 hr (Fig. 6). The average arterial plasma concentration for GTN in control rats was not changed by pretreatment of rats with DPI until 5 min after injection (Fig. 6A). The AUC calculated for GTN in control rats was 2810 ± 868 nM · min and after DPI treatment was 4075 ± 644 nM · min ($P < 0.05$ significant difference compared with control, Student's *t*-test for unpaired data, N = 5). The time courses for arterial plasma concentrations of 1,2-GDN and 1,3-GDN were affected differently by DPI treatment (Fig. 6, B and C). The AUC calculated for 1,2-GDN was 29,600 ± 5,020 nM · min in controls versus 28,900 ± 6,690 nM · min after DPI treatment ($P > 0.05$, Student's *t*-test for paired data, N = 8). The AUC calculated for 1,3-GDN was 27,300 ± 7,270 nM · min in controls versus 40,800 ± 15,600 nM · min after DPI treatment ($P < 0.05$, Student's *t*-test for paired data, N = 8). For ease of comparison to previous data with intact vascular tissue and for comparison to the current *in vitro* biochemical data, the selective effects of DPI on the ratios of plasma 1,2-GDN to 1,3-GDN are shown (Fig. 7).

TABLE 2. Effect of 10 μM DPI on GTN biotransformation mediated by rat aortic microsomes and purified rat liver NADPH-cytochrome P450 reductase

Preparation*	Treatment	1,3-GDN formation† (pmol/min/mg protein)	1,2-GDN formation† (pmol/min/mg protein)
Aortic microsomes	Control	0.63 ± 0.27	1.03 ± 0.53
	10 μM DPI	0.07 ± 0.17‡	0.13 ± 0.17‡
Purified cDNA-expressed NADPH-cytochrome P450 reductase	Control	139 ± 37	99 ± 22
	10 μM DPI	15 ± 25§	10 ± 17§

*Aortic microsomal protein (500 μg of protein/mL; 0.004 U/mL of P450 reductase activity) or purified P450 reductase (5 μg of protein/mL; 0.09 U/mL) were pretreated with NADPH (100 μM) and either DPI (10 μM) or diluent for 30 and 10 min, respectively, prior to the addition of NADPH (1 mM) and GTN (0.2 μM). Incubations were performed under anaerobic conditions at 37° and 1-mL aliquots were taken at 60 min to quantitate dinitrates formed. In two of three experiments using purified enzyme treated with DPI, there were no dinitrates detected. 1 U = enzyme activity equivalent to 1 μmol cytochrome *c* reduced/min.

†Each value represents the mean ± SD (N = 4 for aortic microsomes and N = 3 for purified enzyme).

‡$P < 0.05$, significantly different from the control (Student's *t*-test for paired data).

§$P < 0.01$, significantly different from the control (Student's *t*-test for paired data).

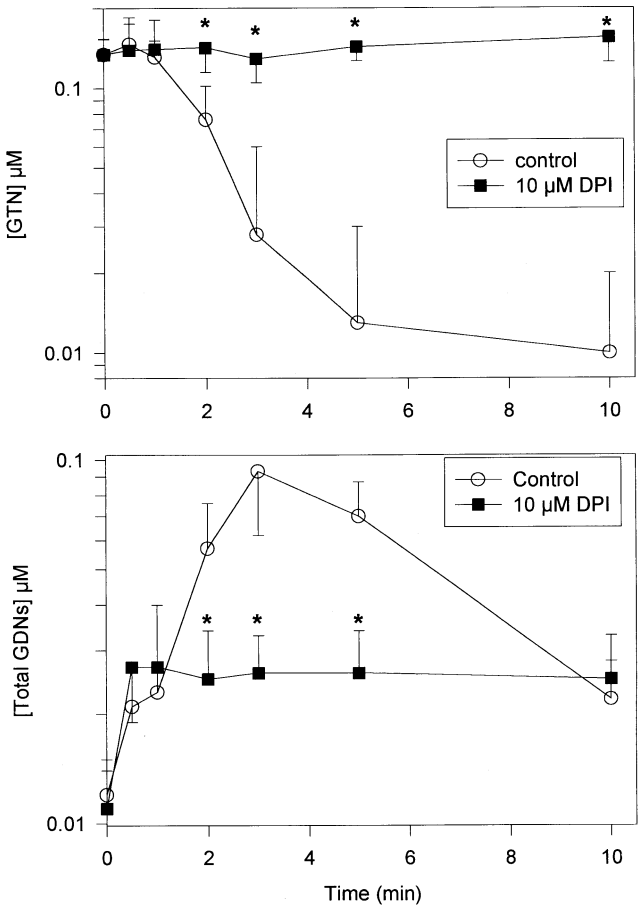

FIG. 3. Effect of pretreatment of hepatic microsomes with DPI on the biotransformation of GTN. Hepatic microsomes (0.9 mg/mL of protein; 0.026 ± 0.002 U/mL of NADPH-cytochrome P450 reductase activity) were pretreated with 100 µM NADPH and either diluent (○) or 10 µM DPI (■) for 10 min prior to exposure to 0.2 µM GTN and 1 mM NADPH. Consecutive aliquots were taken from anaerobic incubations over a 10-min period for quantitation of GTN, 1,3-GDN, and 1,2-GDN. Points on the graphs represent means ± SD (N = 4). *P < 0.05, significantly different from the control (Student's *t*-test for paired data). 1 U = enzyme activity equivalent to 1 µmol cytochrome *c* reduced/min.

DISCUSSION

In this study, we found that cDNA-expressed purified rat liver NADPH-cytochrome P450 reductase catalysed the conversion of GTN to 1,2-GDN and 1,3-GDN, albeit at a lower rate than that observed using hepatic or aortic microsomes when reductase content was accounted for. Consistent with the GTN biotransformation by hepatic and aortic microsomes [6, 7], metabolite formation during incubation of GTN and NADPH-cytochrome P450 reductase occurred under anaerobic but not aerobic conditions. Based on immunoblot analysis and enzyme activity, we estimate that the content of NADPH-cytochrome P450 reductase represents between 0.03 and 0.1% of aortic microsomal protein. We found that DPI, an inhibitor of the action of GTN in isolated rat aorta [18], inhibited the NADPH-cytochrome *c* reductase activity of purified expressed NADPH-cytochrome P450 reductase and that of rat hepatic and aortic microsomes. Inhibition of enzyme activity occurred in a manner that was time- and concentration-dependent, evidence that supports the hypothesis of an irreversible mechanism of enzyme inhibition. DPI also inhibited GTN biotransformation by purified expressed NADPH-cytochrome P450 reductase, aortic microsomes, and hepatic microsomes. Consistent with the inhibition of GTN biotransformation and the hypothesis that the NADPH-cytochrome P450 reductase-cytochromes P450 system mediates bioactivation of GTN to an activator of guanylyl cyclase, DPI inhibited the hepatic microsome-mediated activation of crude soluble guanylyl cyclase by GTN.

The NADPH-cytochrome P450 reductase-mediated formation of GDNs occurred only under anaerobic conditions, suggesting a reductive metabolism of GTN that is inhibited by oxygen. This could be due to competition by oxygen with GTN for electrons supplied by the reductase, since direct one-electron reduction of oxygen by NADPH-cytochrome P450 reductase has been reported [32, 33]. An examination of GTN biotransformation under seemingly unphysiological conditions of low oxygen is not unwarranted when evaluating the biochemical mechanisms of organic nitrate action. It has been observed previously that under reduced oxygen concentrations, the vasodilator response to GTN in isolated rat aorta is enhanced [9], and it was suggested that the preferential venodilator effect of GTN could be due to the lower oxygen tension found in the venous circulation.

The rate of GTN biotransformation by purified NADPH-cytochrome P450 reductase was much less than the rate of NADPH-dependent cytochrome *c* reduction, suggesting that GTN is a poorer substrate than cytochrome *c* for NADPH-cytochrome P450 reductase. When expressed relative to NADPH-cytochrome *c* reductase activity, GTN biotransformation by the purified reductase was considerably less than that of microsomes (Table 2). In aortic and hepatic microsomes, the biotransformation of GTN is NADPH dependent and is inhibited by carbon monoxide [6, 7], evidence that is considered characteristic of cytochromes P450-mediated drug metabolism. Because microsomes contain both components of the mixed-function oxidase system, we conclude that GTN biotransformation occurs at a greater rate when both components of the system are present and that the primary role of the reductase in microsomal GTN metabolism is to supply the electrons from NADPH to cytochromes P450. The DPI-dependent inhibition of hepatic microsome-mediated activation of crude soluble guanylyl cyclase by GTN (Fig. 4) is evidence that supports the hypothesis that the cytochromes P450 system also mediates bioactivation of organic nitrates in liver microsomes. However, the relevance of the cytochromes P450 system in mediating the bioactivation of GTN in vascular tissues is less defined, since incubation of GTN with aortic microsomes does not result in the formation of activators of guanylyl cyclase. Because the rate of

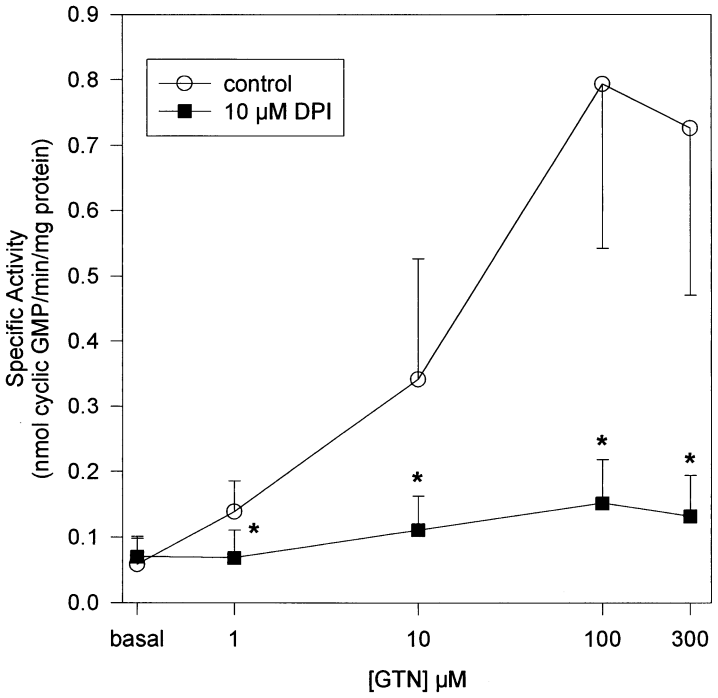

FIG. 4. Effect of pretreatment of hepatic microsomes with DPI on activation of aortic guanylyl cyclase by GTN. Hepatic microsomes were either untreated (○) or exposed to 10 μM DPI (■) for 10 min. Anaerobic incubations contained 20.9 ± 1.8 μg of aortic supernatant protein and 25.6 ± 3.3 μg of microsomal protein (20 pmol cytochrome P450). Points on the graph represent means ± SD (N = 5). *$P < 0.05$, significantly different from the control (Student's *t*-test for paired data).

biotransformation is low relative to that of liver microsomes, aortic microsomal preparations may not generate sufficient NO for activation of guanylyl cyclase under the conditions of the *in vitro* assay employed [9]. Based on the inability to detect CO binding to the heme of cytochromes P450 by classical spectrophotometry in aortic microsomes, and on evidence of low rates of 7-ethoxycoumarin-*O*-deethylase activity in aortic microsomes, there appears to be a lower abundance of constitutive cytochromes P450 present in the aorta than in the liver [6]. We report here that the content of NADPH-cytochrome P450 reductase in aortic microsomes is 5- to 10-fold less than that found in hepatic microsomes. Thus, the difference in rates of GTN biotransformation between aortic and hepatic microsomes may be due to the lower abundance of both components of the cytochromes P450-NADPH-cytochrome P450 reductase system in aorta.

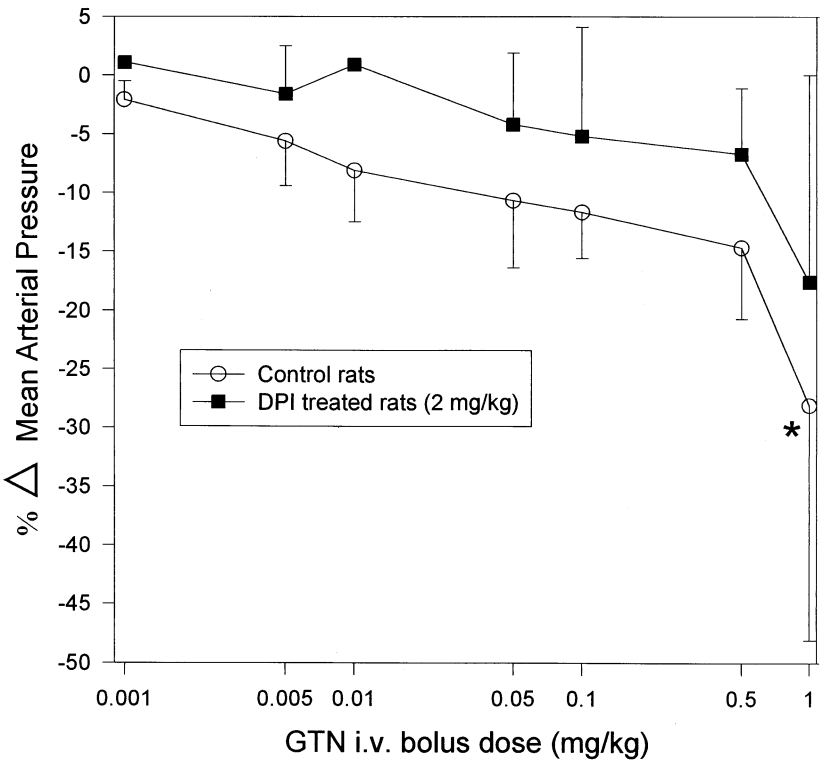

FIG. 5. Effect of DPI on GTN-induced vasodilation *in vivo*. Rats were treated with diluent (○) or DPI (■) for 15 min prior to obtaining blood pressure response curves to GTN (0.001 to 1 mg/kg i.v.) doses. Baseline mean arterial pressures were: control (N = 5), 113 ± 5 mm Hg; DPI treated (N = 5), 104 ± 10 mm Hg. Data points represent means ± SD (N = 5). *P < 0.05, significantly different from the control (repeated measures ANOVA, Newman–Keuls post hoc test).

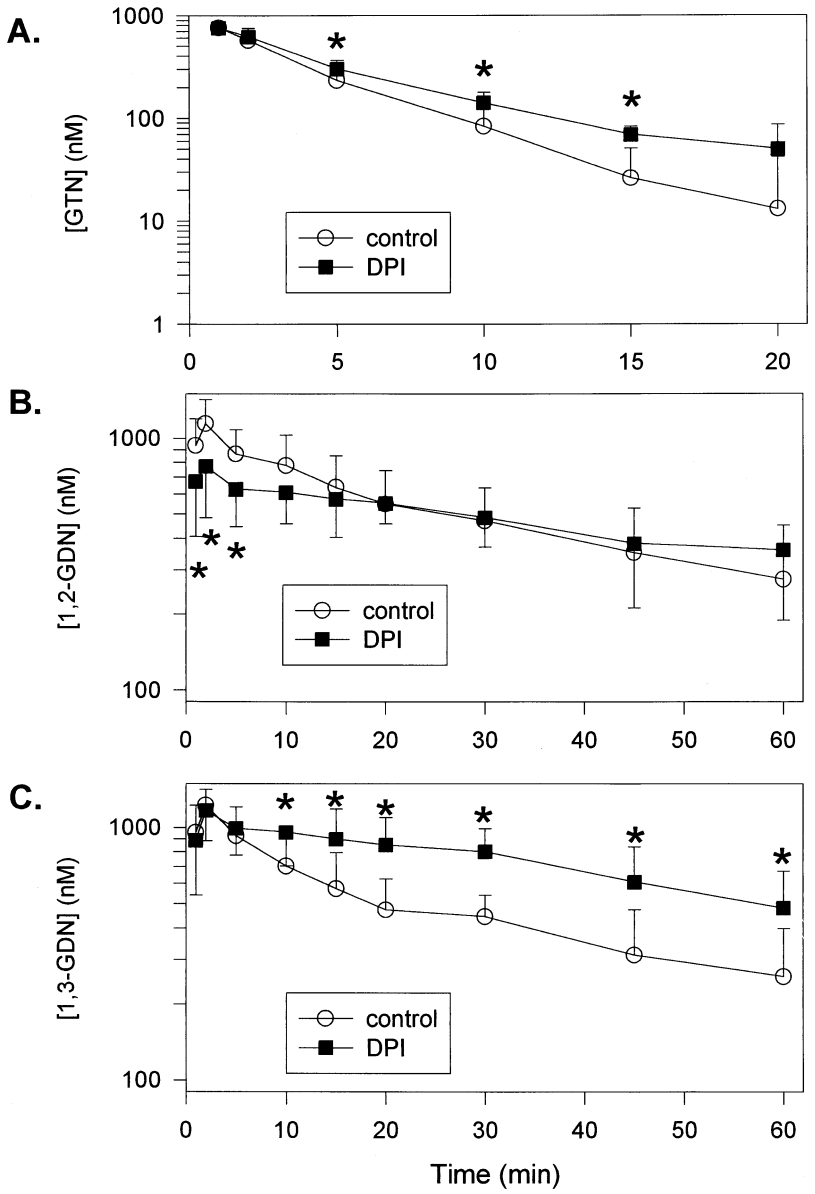

FIG. 6. Effect of DPI on the plasma concentrations of GTN and its dinitrate metabolites after *in vivo* administration. Rats were treated with diluent (○) or DPI (■) for 15 min prior to a single i.v. bolus dose of GTN (1 mg/kg). Arterial blood samples (250 μL) were taken at various time points, and the plasma concentrations of GTN, 1,2-GDN, and 1,3-GDN were determined (see Materials and Methods for details). Data points represent means ± SD (N = 5–8). *$P < 0.05$, significantly different from the control (Student's *t*-test for unpaired data).

DPI is an inhibitor of several flavin-containing enzymes, including neutrophil NADPH-oxidase [13], xanthine oxidase [15], and NO synthases [16, 34]. The cytochromes P450-NADPH-cytochrome P450 reductase system is closely analogous to these enzymes. NADPH-oxidase is also composed of heme (cytochrome b_{558}) and flavin (FAD) redox centers [14], and NO synthases are hemeproteins containing both FAD and FMN [35]. Although it is possible that DPI may react with other microsomal flavoproteins, the inhibition of NADPH-cytochrome *c* reductase activity in microsomes is accompanied by a similar degree of inhibition of GTN biotransformation. At the present time, there is only one reference to other GTN-metabolizing enzymes in microsomes. Chung *et al.* [8] reported the partial isolation of a microsomal protein from bovine coronary artery that produced detectable levels of NO after incubation with GTN. Whether this GTN-metabolizing enzyme is inhibited by DPI or is present in rat aorta has not been reported. It is noteworthy that DPI had no effect on GTN-induced relaxation of bovine coronary artery, suggesting that if this enzyme is present in rat aorta, it is not the target for DPI inhibition of GTN action [36].

We tested the effect of DPI on NADH-cytochrome b_5 reductase, another flavoprotein present in aortic and hepatic microsomes. Although NADH-cytochrome b_5 reductase shares some homology to NADPH-cytochrome P450 reductase through their $NADP^+$-ferredoxin reductase-like domains [37], it was not inhibited by DPI. This is in contrast to the inhibition of the mitochondrial FAD-containing Complex 1 (NADH-ubiquinone oxidoreductase). These results confirm that, in agreement with previous studies, DPI acts via enzyme-catalyzed reactions, rather than as a non-specific inhibitor of all flavin-containing proteins. We further tested whether these enzymes or other flavin-containing enzymes might mediate GTN biotransformation, using NADH as the electron donor. We found

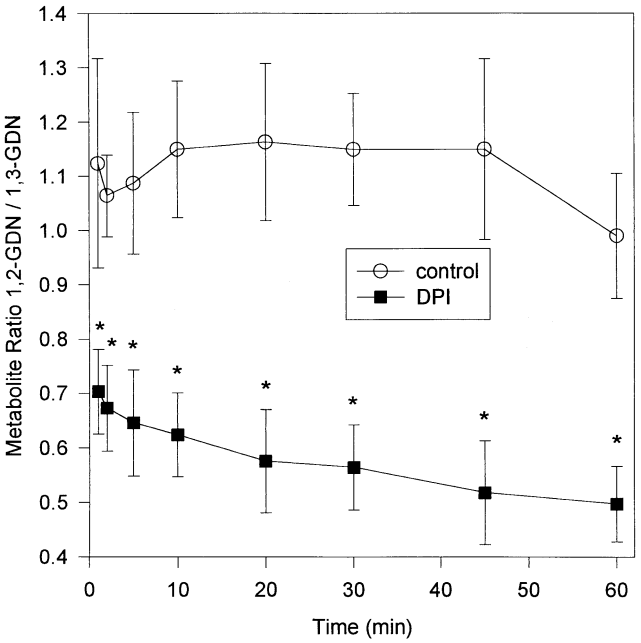

FIG. 7. Time course of the effect of DPI on the arterial plasma metabolite ratios of 1,2-GDN to 1,3-GDN. A comparison of the ratios of 1,2-GDN to 1,3-GDN present in arterial plasma at different time points for rats treated with diluent (○) or DPI (■) for 15 min prior to a single i.v. bolus dose of GTN (1 mg/kg). Points on the graph represent means ± SD (N = 7–8). *$P <$ 0.01, significantly different from the control (Student's *t*-test for unpaired data).

that although NADH did support GTN biotransformation by hepatic microsomes (anaerobic) and hepatic mitochondria (aerobic), there was no production of activators of aortic guanylyl cyclase by these preparations, suggesting the absence of a role for NADH-dependent enzymes in mediating GTN-induced relaxation. The identities of the liver enzymes responsible for the novel observation of NADH-dependent GTN biotransformation are currently unknown. In mitochondria, based on the oxygen dependence, inhibition by DPI, and inhibition by carbon monoxide, the data suggest the participation of the electron transport chain in NADH-dependent GTN biotransformation. The lack of detectable GTN biotransformation by aortic mitochondria suggests that DPI inhibition of mitochondria in isolated aorta would likely not result in measurable changes in the biotransformation activity of this tissue. This is consistent with a recent study demonstrating that the competitive mitochondrial inhibitors rotenone and amytal had no effect on GTN-induced relaxation in isolated aorta [38].

There are reports of NADPH- and NADH-dependent oxidase activities in homogenized rabbit aorta and in cultured rat vascular smooth muscle cells that are stimulated by treatment with angiotensin II and inhibited by DPI [39–42]. Furthermore, after differential centrifugation of the homogenate, the oxidase activities, as measured by lucigenin chemiluminescence, were found to migrate with the plasma membrane-enriched particulate fraction rather than the cytosolic fraction [40]. The relevance of these oxidase activities to the current study is two-fold. If the enzymes responsible for the oxidase activities are analogous to the NADPH-oxidase system of macrophages and neutrophils, then they represent a possible target for DPI in the aorta. Also, it was suggested that development of tolerance to GTN is associated with increased superoxide production mediated by vascular NADPH- and NADH-oxidases, leading to decreased activation of guanylyl cyclase by nitrovasodilators due to quenching of NO by superoxide [41]. GTN-tolerant aortic rings from rabbits treated *in vivo* with transdermal GTN patches demonstrated increased superoxide production that was inhibited by treatment of the rings with DPI but not by inhibitors of mitochondrial NADH-ubiquinone oxidoreductase, xanthine oxidase, cyclooxygenase, or NO synthases [41]. However, an effect of DPI to inhibit O_2^- production by NADPH/NADH oxidase would be expected to enhance GTN-induced vasodilation rather than inhibit it, as has been observed previously [18]. Thus, although aortic NADPH/NADH oxidase may be a target for DPI, the inhibitory effect of DPI on this enzyme would appear to be unrelated to its inhibitory effect on GTN-induced vasodilation.

A role for endothelial NO synthase or for xanthine oxidase in the inhibitory effects of DPI on aortic microsomal GTN biotransformation is unlikely, because aortae were denuded of endothelium prior to preparation of microsomes, and since xanthine oxidase is a cytosolic enzyme. In addition, DPI has no effect on the cytosolic biotransformation of GTN, the majority of which is mediated by glutathione *S*-transferases [3, 19]. Finally, there is the possibility that FMOs participate in GTN metabolism and are inhibited by DPI. However, there is no evidence that there are constitutive aortic FMOs or that FMOs react with DPI or GTN. In addition, carbon monoxide inhibits microsomal GTN biotransformation in aorta and liver [6, 7] but does not inhibit FMO-mediated drug metabolism [43].

The excellent fit of the activity data to a model of pseudo-first-order inactivation kinetics indicates that the microsomal NADPH-cytochrome P450 reductase behaves the same as the purified enzyme with respect to the inhibitory mechanism of DPI. The correlation of residual activity with the time of preincubation of enzyme with DPI and NADPH indicates that enzyme turnover is necessary for inhibition of enzyme activity. Based on the correlation between k_3 values and concentrations of DPI, there was an extremely good fit of the experimental data with that expected for an enzyme that binds an inhibitor by Michaelis–Menten kinetics. Taken together, the kinetic analysis is consistent with the notion that DPI is a mechanism-based inactivator of NADPH-cytochrome P450 reductase. The differences in apparent K_i values between liver microsomes and the other two preparations suggest that the binding affinity of DPI for reductase in the liver microsomes is different. The reason for this difference is unclear but may be due to differences in access of DPI to the initial binding site on NADPH-cytochrome P450 reductase and/or an additional target in liver microsomes, e.g. the unknown

NADH-dependent enzyme capable of GTN biotransformation.

Although the sites of irreversible modification of NADPH-cytochrome P450 reductase by DPI remain to be determined, the lack of inhibition by DPI of the homologous flavoproteins, NADH-cytochrome b_5 reductase (reported herein), and glutathione reductase [44] may provide some insight into the nature of the modification. Two sites of modification of NADPH-cytochrome P450 reductase by the DPI analog IDP have been proposed to contribute to inhibition of enzyme activity. Using mass spectrometry, it was shown that phenylated flavins were formed after inhibition of the enzyme by IDP. Also, HPLC analysis of trypsin digested *S*-carboxymethylated enzyme identified Trp 419 as being labelled by [^{14}C]IDP [17]. The modification of an amino acid in this region corresponds to the so-called "insertion sequence" that is absent in both NADH-cytochrome b_5 reductase and glutathione reductase since they each contain only one flavin. This sequence is proposed to orient the position of the two flavin binding domains of P450 reductase for electron transfer between FAD and FMN [45]. In preliminary studies, we found that purified NADPH-cytochrome P450 reductase is labelled by [^{125}I]DPI in an NADPH-dependent manner [46], but further work is necessary to determine whether DPI forms adducts with P450 reductase at this particular amino acid site. Autoradiographic studies using neutrophil membrane preparations of NADPH-oxidase have shown that [^{125}I]DPI forms covalent adducts with the polypeptide chains of the flavin-containing subunits, and of cytochrome b_{558} of this enzyme complex [13, 14]. Given the close association of cytochrome P450 with NADPH-cytochrome P450 reductase, it is possible that cytochrome P450 may also be a site of modification for DPI radicals formed by the reductase. In preliminary studies, we found that incubation of [^{125}I]DPI with aortic microsomes results in labelling of 79-kDa and 50-kDa proteins, which would be consistent with the binding of DPI to both P450 reductase and cytochrome P450 [46].

To evaluate the *in vivo* significance of DPI-mediated inhibition of GTN action found in the current study and that observed in isolated blood vessels [18], we tested the effect of DPI on the pharmacodynamics and pharmacokinetics of GTN in conscious rats. Consistent with the inhibition by DPI of GTN-induced relaxation of isolated blood vessels [18], DPI treatment of rats resulted in inhibition of GTN-induced vasodilation *in vivo*. Wang and Pang [47] reported that *in vivo* administration of DPI causes a very rapid release of norepinephrine into the circulation that significantly increases MAP for about 5 min. In our protocols, rats were administered DPI 15 min prior to GTN, at which point heart rate and MAP had returned to control values.

Inhibition of the blood pressure response to the highest dose of GTN (1 mg/kg) was modest, but was complementary to the effect of DPI on altering the pharmacokinetics of an equal dose of GTN. The disappearance of GTN from rat arterial plasma is very rapid, and DPI had a small but significant effect on increasing the half-life and increasing the AUC for GTN (Table 3), as well as effects on the appearance of dinitrate metabolites. Specifically, the initial phase of rapid appearance of 1,2-GDN in the plasma (0–5 min) was inhibited significantly by pretreatment of rats with DPI (Fig. 6B), whereas the initial appearance of 1,3-GDN was unaffected by DPI (Fig. 6C). It is during a very short time period (30 sec–1 min) after bolus i.v. GTN injection that mean arterial pressure is lowered in conscious rats. This difference in regioselective GTN denitration after DPI treatment was manifested in a decrease in the 1,2-GDN to 1,3-GDN ratio during the entire 1-hr time course examined (Fig. 7). It is noteworthy that selective formation of 1,2-GDN from GTN has been demonstrated in isolated blood vessels and cell lines exposed to low concentrations of GTN and short time periods of incubation, and that selective 1,2-GDN formation occurs after incubation of aortic microsomes (Table 2, [7]). In addition, the selective 1,2-GDN formation from GTN observed in isolated blood vessels is absent in tissues made tolerant to GTN [20]. Finally, the selective inhibition of 1,2-GDN formation by DPI also occurs in isolated blood vessels [18]. All of the above data are consistent with the notion that selective 1,2-GDN formation, both *in vivo* and *in vitro*, is indicative of mechanism-based biotransformation, and is mediated by a DPI-inhibitable process.

TABLE 3. Summary of pharmacokinetic parameters for GTN in conscious rats pretreated with vehicle or DPI

	C_{max} (nM)	Half-life (min)	AUC (nM · min)
Control rats	727 ± 171	3.3 ± 1.1	2810 ± 868
DPI-treated rats	753 ± 118	5.0 ± 1.0*	4075 ± 644*

Pharmacokinetic parameters were determined by fitting the GTN arterial plasma concentrations from individual rats pretreated with either vehicle or DPI, to a first-order kinetic model. Values are means ± SD (N = 5).

*$P < 0.05$, significantly different from the control (Student's *t*-test for unpaired data).

It should be noted that the regioselective inhibition of 1,2-GDN formation by DPI observed in intact cells was absent in experiments using microsomes or purified P450 reductase (both 1,2-GDN and 1,3-GDN formation was inhibited to a similar degree). Whereas this might argue that selective 1,2-GDN formation is not relevant, it must be appreciated that regioselective 1,2-GDN formation is itself much attenuated in broken cell preparations. It is therefore difficult to draw conclusions regarding regioselective GTN biotransformation from data obtained in broken cell preparations.

After the initial phase (1–5 min) of dinitrate appearance, the arterial plasma concentrations of 1,3-GDN were significantly greater in rats treated with DPI than in controls, whereas the levels of 1,2-GDN appeared to be the same in control and DPI-treated rats. Because multiple biotransformation pathways for organic nitrates exist, it is likely that

the increased 1,3-GDN formation in the presence of DPI reflects diversion of GTN biotransformation to other pathways that exhibit regioselectivity for 1,3-GDN formation (e.g. GSTs).

In summary, based on kinetic analyses of enzyme inhibition by DPI, we have shown that DPI is a mechanism-based inactivator of NADPH-cytochrome P450 reductase. These data do not imply that NADPH-cytochrome P450 reductase is the only target for DPI in isolated aorta, but do support our hypothesis that vascular NADPH-cytochrome P450 reductase is a site of action for the irreversible inhibition by DPI of the metabolic activation of GTN. The *in vivo* data provide further evidence to support the idea that mechanism-based GTN biotransformation is mediated by an enzyme system selective for 1,2-GDN formation and sensitive to inhibition by DPI.

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